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Pluripotent Cell Lines Derived from Common Marmoset (*Callithrix jacchus*) Blastocysts¹

James A. Thomson,^{2,3} Jennifer Kalishman,³ Thaddeus G. Golos,^{3,4} Maureen Durning,³ Charles P. Harris,⁶ and John P. Hearn^{3,5}

The Wisconsin Regional Primate Research Center,³ Departments of Obstetrics and Gynecology⁴ and Physiology,⁵ School of Medicine, and Cytogenetics Laboratory,⁶ State Hygiene Laboratory, University of Wisconsin, Madison, Wisconsin 53715–1299

ABSTRACT

We report the derivation of eight pluripotent cell lines from common marmoset (*Callithrix jacchus*) blastocysts. These cell lines are positive for a series of markers (alkaline phosphatase, SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81) that characterize undifferentiated human embryonal carcinoma cells and rhesus embryonic stem cells. All eight cell lines had a modal chromosome number of 46; seven cell lines were XX and one was XY. Two cell lines (Cj11 and Cj62) were cultured continuously for over a year and remained undifferentiated and euploid. In the absence of fibroblast feeder layers, these cell lines differentiated to multiple cell types, even in the presence of leukemia inhibiting factor. Differentiated cells secreted bioactive CG into the culture medium and expressed α -CG, β -CG, and α -fetoprotein mRNA, indicating trophoblast and endoderm differentiation. Bioactive CG secretion in differentiating cells was increased substantially in the presence of GnRH agonist D-Trp⁶-Pro⁹-NH₂. When grown at high densities, these cells formed embryoid bodies with a close resemblance to early postimplantation embryos, including the formation of a yolk sac, amnion, and an embryonic disc with an early primitive streak. These results make these pluripotent cells strong candidates for marmoset embryonic stem cells.

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cell lines capable of contributing to derivatives of all three embryonic germ layers even after prolonged culture [1–3]. Mouse ES cells in chimeras sometimes contribute to germ cells, thus providing a vehicle for introducing genetic changes into the germ line [4]. Because homologous recombination allows the alteration of specific loci of the genome, mouse ES cells allow the production of very specific models of human genetic diseases [5]. However, because of the differences between human and mouse development, anatomy, and physiology, transgenic mice can provide only a limited understanding of some human diseases. In addition, the testing of new therapies in transgenic mice is limited by mouse size, life span, and physiology. Transgenic primate models would increase our understanding of the pathogenesis of specific diseases and allow the testing of new therapies. In transgenic primates, therapeutic efficacy for treating degenerative neural diseases, such as Alzheimer's disease, could

be assessed not only by morphological and biochemical changes in the brain, but by changes in complex behaviors.

We have recently reported the isolation of ES cells from the rhesus monkey that are immortal, have a stable normal karyotype, and have the potential to differentiate to derivatives of trophoblast and all three embryonic germ layers [6]. Rhesus monkey ES cells provide a powerful new in vitro model for understanding the differentiation of human tissues, but the reproductive biology of rhesus monkeys makes testing the ability of these cells to contribute to the germ line in chimeras impractical. The rhesus monkey, which is an Old World primate species, has single young, reaches sexual maturity at 4–5 yr, and has an ovarian cycle that cannot be routinely synchronized. The common marmoset, a New World primate species, has more favorable reproductive characteristics for experimental primate embryology, including the natural birth of twins or triplets, an early age at sexual maturity (about 18 mo), and an ovarian cycle that can be synchronized with prostaglandins, thus allowing efficient embryo collection and transfer [7–9].

Here we report the derivation of eight pluripotent cell lines from common marmoset blastocysts that closely resemble rhesus ES cells and human embryonal carcinoma (EC) cells in morphology, growth characteristics, cell surface markers, and in vitro differentiation. Because of the reproductive characteristics of the common marmoset, it will be possible to define the developmental potential of these pluripotent cell lines in chimeras with normal embryos in vivo, initiating exciting advances in experimental primate embryology.

MATERIALS AND METHODS

Embryo Recovery and Cell Line Isolation

For embryo donors, female marmosets greater than 2 yr of age and demonstrating regular ovarian cycles were maintained in groups with a fertile male and up to five progeny. Ovarian cycles were controlled by i.m. injection of 0.75 μ g of the prostaglandin F_{2 α} analog cloprostenol (Estrumate; Mobay Corp., Shawnee, KS) during the middle to late luteal phase [7]. Blood samples (0.2 ml) were collected in heparinized syringes on Day 0 (immediately before cloprostenol injection), and on Days 3, 7, 9, 11, and 13. Plasma progesterone concentrations were determined by ELISA [10]. The day of ovulation was taken as the day preceding a plasma progesterone concentration of 10 ng/ml or more [8]. Eight days after ovulation, marmosets were lightly anesthetized by the i.m. injection of alphaxalone and alphadolone (Saffan; Glaxovet, Ltd., Uxbridge, UK), and blastocysts were recovered by a nonsurgical uterine flush procedure [9].

Blastocysts were incubated in 0.5% pronase-Dulbecco's

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²Correspondence: James Thomson, The Wisconsin Regional Primate Research Center, University of Wisconsin, 1223 Capitol Court, Madison, WI 53715–1299. FAX: (608) 263–3524.

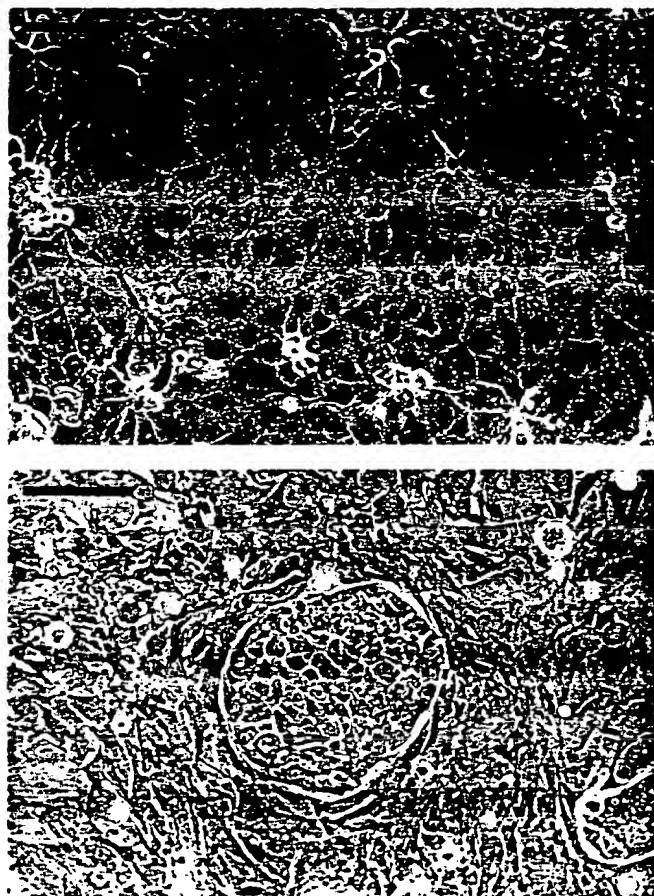


FIG. 1. Colony morphology and in vitro differentiation of cell line Cj62. A) Undifferentiated Cj62 cells on a background of embryonic fibroblasts. Note the distinct colony border, high nucleus:cytoplasm ratio, and prominent nuclei (bar = 100 μ M). B) Differentiated cells 8 days after Cj62 cells were plated on gelatin-treated tissue culture plastic, with 10^5 U/ml added human LIF (bar = 100 μ M).

removed from fibroblast feeders, they differentiated into cells of several distinct morphologies, even in the presence of human LIF (Fig. 1B). The cells also differentiated when allowed to grow beyond confluence on fibroblast feeder layers. Among the differentiated cells derived from Cj11 and Cj62, trophoblast was indicated by the expression of the Cg α and Cg β mRNAs detected by RT-PCR (Fig. 3), and by the secretion of bioactive CG into the culture medium (Fig. 4). Differentiated cells also expressed mRNA for GnRH (Fig. 3), and the secretion of bioactive CG increased substantially when differentiating cells were exposed to GnRH agonist (Fig. 4). Endoderm differentiation (probable extra-embryonic endoderm) was indicated by the presence of α FP mRNA, detected by RT-PCR (Fig. 3). When each of the eight pluripotent marmoset cell lines was grown at high density, over a period of 1–2 wk epithelial cells differentiated and covered the culture dish; the remaining groups of undifferentiated cells contracted into compact balls and then formed embryoid bodies. Over 3–4 wk, some of the embryoid bodies formed a bilaterally symmetric pyriform embryonic disc, an amnion, a yolk sac, and a mesoblast outgrowth attaching the caudal pole of the amnion to the culture dish. Histological and ultrastructural examination of one of these embryoid bodies (formed from a cell line, Cj62, that had been passaged continuously for 6 mo) revealed a close resemblance to an early primitive

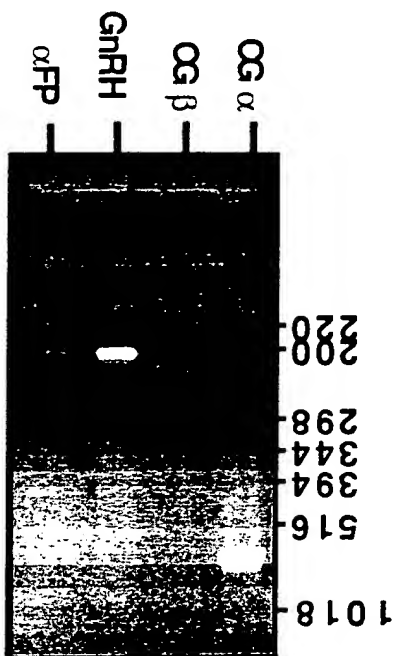
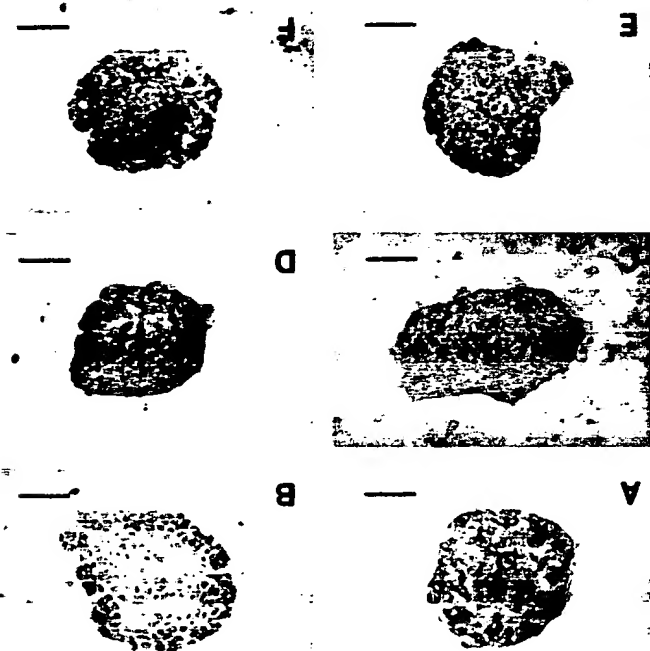


FIG. 3. RT-PCR amplification of mRNAs for Cg α , Cg β , GnRH, and α FP from total RNA from pluripotent marmoset cells allowed to differentiate in vitro. Identities of all cDNAs were confirmed by subcloning and sequencing. Negative controls, which contained no template DNA, produced no bands (not shown).

FIG. 2. Expression of cell surface markers by undifferentiated Cj62 cells (bar = 100 μ M). A) Alkaline phosphatase (Vector Blue substrate). Because no counterstain was used, the fibroblast feeder layer is not visible. B) SSEA-1. C) SSEA-3. D) SSEA-4. E) TRA-1-60. F) TRA-1-81. For panels B–F, detection was with horseradish peroxidase/diaminobenzidine, and positive cells are brown. Counterstaining was with hematoxylin. Although consistently positive, SSEA-3 and TRA-1-81 staining of Cj62 cells was weaker than SSEA-4 and TRA-1-61 and TRA-1-61 staining intensity varied within and between colonies.



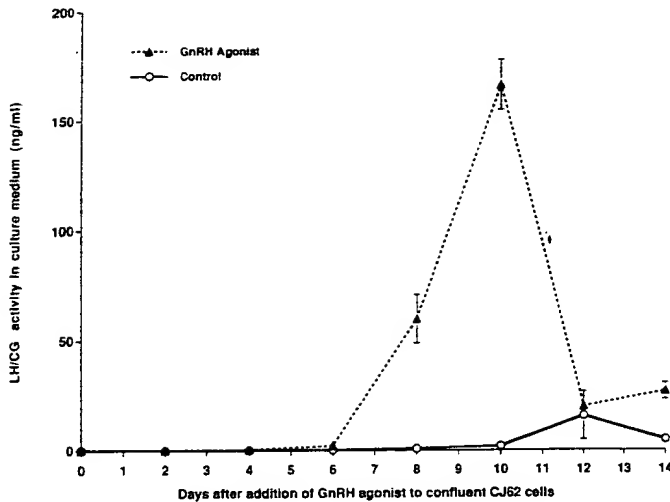


FIG. 4. GnRH agonist D-Trp⁶-Pro⁹-NH₂Et responsiveness of CG secretion in differentiating Cj62 cells. LH/CG bioactivity was measured by Leydig cell bioassay in culture medium conditioned by differentiating Cj62 cells. GnRH agonist was added to undifferentiated Cj62 cells at confluence (Day 0); medium was changed every 2 days and supplemented with fresh agonist. Bars represent SEM.

streak-stage embryo (Fig. 5). The embryonic disc was composed of a polarized, columnar epithelial epiblast (primitive ectoderm) layer separated from a hypoblast (primitive endoderm) layer. Electron microscopy of the epiblast revealed apical junctional complexes, apical microvilli, subapical intermediate filaments, and a basement membrane separating

the epiblast from underlying endoderm—all features of the normal embryonic disc. In the caudal third of the embryonic disc, there was a midline groove, disruption of the basement membrane, and mixing of epiblast cells with underlying endoderm cells (early primitive streak; Fig. 5). An amnion was composed of an inner squamous (ectoderm) layer continuous with the epiblast, and an outer mesoderm layer.

DISCUSSION

Our criteria for ES cells are as follows: derivation from the preimplantation embryo, immortality, a normal karyotype, and the maintained ability to differentiate to derivatives of all three embryonic germ layers. Contribution to the germ line in chimeras is also a property of some mouse ES cell lines, but originally the term was introduced to distinguish the origin of pluripotent mouse cell lines derived from preimplantation embryos (ES cells) from those derived from teratocarcinomas (EC cells) [3]. Although mouse ES and EC cells are very similar, ES cells generally have a greater developmental potential, a difference thought to be related to the selective pressures of the teratocarcinoma environment that are avoided by the *in vitro* derivation of ES cells [3].

Several characteristics of the pluripotent marmoset cell lines we have isolated make them strong candidates for ES cells. First, the pluripotent marmoset cells continue to proliferate rapidly for at least 18 mo in continuous culture, and at least some maintain a normal karyotype. Although spontaneously immortal cell lines have been derived from primary cultures of mouse cells, this occurs rarely, if ever,

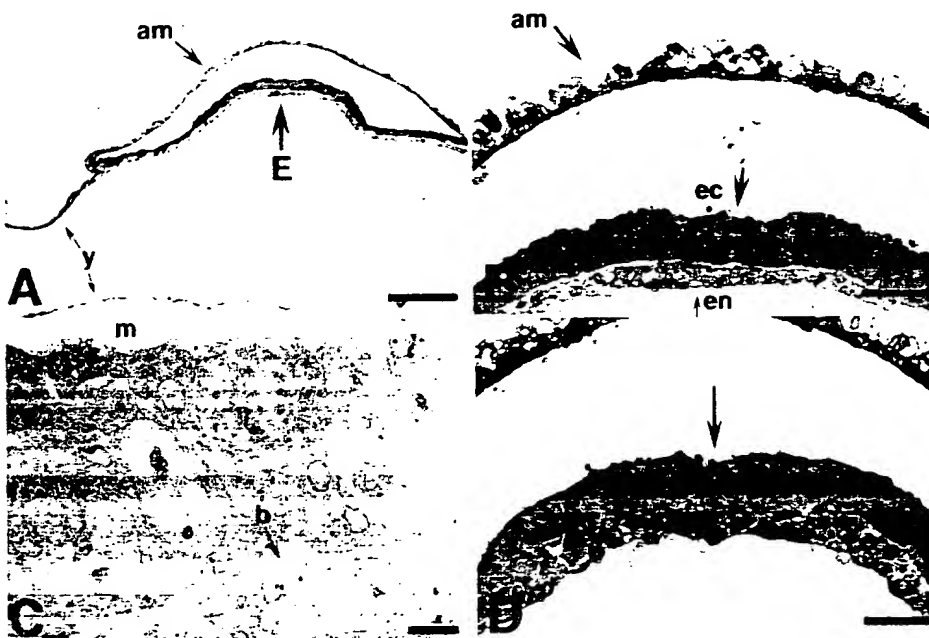


FIG. 5. Embryoid body formed from cell line Cj62 after 6 mo of undifferentiated culture. Cj62 cells were grown to confluence and then were allowed to spontaneously differentiate for 4 wk. A) Structures with morphological characteristic of yolk sac (y), amnion (am), and bilayered embryonic disc (E). The yolk sac was spherical, but collapsed during embedding, and the portion of the yolk sac to the right of the photograph was trimmed so the block would fit the diamond knife for sectioning. The embryonic disc was pyriform-shaped with a central groove in its caudal (narrow) aspect, and was connected to the tissue culture plate at its caudal pole by a stalk of mesenchymal cells. (Bar = 200 μ M, toluidine blue stain). B) Section in cranial 1/3 of embryonic disc. Note that the primitive ectoderm (ec) forms a distinct cell layer from the underlying primitive endoderm (en), with no mixing of cell layers. Note also that the amnion (am) is composed of two distinct layers; the inner layer is continuous with the primitive ectoderm at the margins. (Bar = 50 μ M, toluidine blue stain). C) Electron micrograph of embryonic disc. Apical microvilli (m) and apical junctional complexes (j) are present in the ectoderm layer, and the basement membrane (b) separates the ectoderm from the underlying endoderm. (Bar = 5 μ M, lead citrate and uranyl acetate). D) Section in caudal 1/3 of embryonic disc. Note the central groove (arrow) and the mixing of primitive ectoderm and endoderm. This is the approximate level of early primitive streak formation in the normal primate embryo. (Bar = 50 μ M, toluidine blue stain).

from primary cultures of somatic cells of primates, which consistently undergo crisis after a characteristic number of cell divisions [24]. Our success in isolating multiple immortal cell lines from both rhesus monkey and marmoset ICMs suggests that, unlike adult somatic cells, the undifferentiated, totipotent cells of the early embryo are immortal; that is, they are capable of unlimited proliferation. A second characteristic of the pluripotent marmoset cells is the expression of SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase—a combination of cell surface markers previously described only for rhesus monkey ES cells and human EC cells [6, 14–16, 18]. The differentiation of human EC cells results in the loss of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 expression, and the earliest lineages to differentiate from the ICM in the human embryo, extra-embryonic endoderm and trophoblast, lack this combination of markers [18]. A third important characteristic of the pluripotent marmoset cells is the potential to differentiate to both endoderm and trophoblast, as the last cells in the mammalian embryo capable of contributing progeny to both these lineages are the totipotent early ICM cells [25]. And finally, the pluripotent marmoset cells differentiate to embryoid bodies with a remarkable resemblance to postimplantation early primitive streak-stage embryos [26].

Pluripotent marmoset cells offer an important new *in vitro* model for studying the differentiation and function of tissues that differ significantly between mice and primates. For example, the structure and the function of the trophoblast, which forms the outer layer of the placenta, differs dramatically between primates and rodents. Trophoblast secretion of CG in primates, including humans, is central to the maternal recognition of pregnancy. The mouse placenta does not express a CG, and mouse ES cells fail to differentiate to trophoblast or do so infrequently [27]. If the primate corpus luteum is exposed to CG, progesterone secretion is continued and pregnancy is maintained; in the absence of CG, the corpus luteum regresses, progesterone secretion declines, and a new ovarian cycle is initiated. GnRH is expressed in the placenta and has been proposed to have a local regulatory role in CG secretion [28]. The increase in CG secretion we observed in differentiating pluripotent marmoset cells in the presence of GnRH agonist supports a role in CG expression. Further, the dramatic effects observed suggest that GnRH may not only act on differentiated trophoblasts but might also be directly involved in the differentiation of trophoblasts. GnRH has been shown to be expressed and have biological effects in extrapituitary tissues other than the placenta [29–31], and this may point to a wider role for this regulatory peptide in differentiation and development. Because these pluripotent marmoset cells can be grown indefinitely, prior to differentiation it will be possible to use homologous recombination to modify trophoblast-specific genes, such as CG, GnRH, or their receptors, to help elucidate their function and regulation during and after differentiation.

The pluripotent marmoset cells initiate the formation of all three germ layers in embryoid bodies. If culture conditions can be established that allow efficient, synchronous development of organized embryoid bodies, then it will be possible to use these pluripotent marmoset cells to genetically dissect *in vitro* the mechanisms controlling early primitive streak formation in primates. We are not aware of primitive streak formation occurring in mouse embryoid bodies, which exhibit a more disorganized development [32]. With our present culture conditions, however, em-

bryoid body formation is asynchronous, and many embryoid bodies develop into simple multilayered vesicular structures without the well-organized structure represented in Figure 5. To date, we have not observed development of embryoid bodies beyond the initiation of primitive streak formation, which is also the approximate stage where intact marmoset embryos degenerate in our culture conditions. To rigorously test the developmental potential of these pluripotent marmoset cells, it will be necessary to provide them with a normal embryonic environment, in chimeras with intact embryos.

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REFERENCES

- Martin G. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 1981; 78:7634–7638.
- Evans M, Kaufman M. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; 292:154–156.
- Rossant J, Papaioannou V. The relationship between embryonic, embryonal carcinoma and embryo-derived stem cells. *Cell Differ* 1984; 15:155–161.
- Bradley A, Evans M, Kaufman M, Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* 1984; 309:255–256.
- Rossant J, Bernelot Moens C, Nagy A. Genome manipulation in embryonic stem cells. *Philos Trans R Soc Lond Biol* 1993; 339:207–215.
- Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP. Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci USA* 1995; 92:7844–7848.
- Summers PM, Wennink CJ, Hodges JK. Cloprostenol-induced luteolysis in the marmoset monkey (*Callithrix jacchus*). *J Reprod Fertil* 1985; 73:133–138.
- Lopata A, Summers P, Hearn J. Births following the transfer of cultured embryos obtained by *in vitro* and *in vivo* fertilization in the marmoset monkey (*Callithrix jacchus*). *Fertil Steril* 1988; 50:503–509.
- Thomson JA, Kalishman J, Hearn JP. Non-surgical uterine stage preimplantation embryo collection from the common marmoset. *J Med Primatol* 1994; 23:333–336.
- Saltzman W, Schultz-Darken NJ, Scheffler G, Wegner FH, Abbott DH. Social and reproductive influences on plasma cortisol in female marmoset monkeys. *Physiol Behav* 1994; 56:801–810.
- Solter D, Knowles B. Immunosurgery of mouse blastocysts. *Proc Natl Acad Sci USA* 1975; 72:5099–5102.
- Robertson EJ. Embryo-derived stem cell lines. In: Robertson EJ (ed.). *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. Washington, DC: IRL Press; 1987: 71–112.
- Hsu T, Benirschke K. *An Atlas of Mammalian Chromosomes*. New York: Springer-Verlag; 1970.
- Solter D, Knowles BB. Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc Natl Acad Sci USA* 1978; 75:5565–5569.
- Kannagi R, Cochran NA, Ishigami F, Hakomori S, Andrews PW, Knowles BB, Solter D. Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *EMBO J* 1983; 2:2355–2361.
- Andrews PW, Banting G, Damjanov I, Arnaud D, Avner P. Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. *Hybridoma* 1984; 3:347–361.
- Andrews P, Damjanov I, Simon D, Banting G, Carlin C, Dracopoli

- N. Fogh J. Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. *Lab Invest* 1984; 50:147-162.
18. Andrews P, Oosterhuis J, Damjanov I. Cell lines from human germ cell tumors. In: Robertson E (ed.), *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. Oxford: IRL Press; 1987: 207-246.
19. Golos TG, Durning M, Fisher JM, Fowler PD. Cloning of four growth hormone/chorionic somatomammotropin-related complementary deoxyribonucleic acids differentially expressed during pregnancy in rhesus monkey placenta. *Endocrinology* 1993; 133:1744-1752.
20. Adelman JP, Mason AJ, Hayflick JS, Seeburg PH. Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. *Proc Natl Acad Sci USA* 1986; 83:179-183.
21. Vale W, Rivier C, Brown M, Leppaluoto J, Monahan M, Rivier J. Pharmacology of hypothalamic regulatory peptides. *Clin Endocrinol* 1976; 5:261.
22. Terasawa E, Bridson WE, Nass TE, Noonan JJ, Dierschke DJ. Developmental changes in the luteinizing hormone secretory pattern in peripubertal female rhesus monkeys: comparisons between gonadally intact and ovariectomized animals. *Endocrinology* 1984; 115:2233-2240.
23. Andrews PW. Human teratocarcinomas. *Biochim Biophys Acta* 1988; 948:17-36.
24. Shay JW, Wright WE, Werbin H. Defining the molecular mechanisms of human cell immortalization. *Biochim Biophys Acta* 1991; 1072: 1-7.
25. Winkel GK, Pedersen RA. Fate of the inner cell mass in mouse embryos as studied by microinjection of lineage tracers. *Dev Biol* 1988; 127:143-156.
26. Lopata A, Berka J, Simula A, Norman R, Otani T. Differential distribution of mRNA for the α - and β -subunits of chorionic gonadotropin in the implantation stage blastocyst of the marmoset monkey. *Placenta* 1995; 16:335-346.
27. Beddington RSP, Robertson EJ. An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* 1989; 105:733-737.
28. Siler-Khodr TM, Khodr GS. Luteinizing hormone releasing factor content of the human placenta. *Am J Obstet Gynecol* 1978; 130:216-219.
29. Peng C, Fan NC, Ligier M, Vaananen J, Leung PCK. Expression and regulation of gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells. *Endocrinology* 1994; 135:1740-1746.
30. Whitelaw PF, Eidne KA, Sellar R, Smyth CD, Hillier SG. Gonadotropin-releasing hormone receptor messenger ribonucleic acid expression in rat ovary. *Endocrinology* 1995; 136:172-178.
31. Olofsson JI, Conti CC, Leung PCK. Homologous and heterologous regulation of gonadotropin-releasing hormone receptor gene expression in preovulatory rat granulosa cells. *Endocrinology* 1995; 136: 974-980.
32. Doetschman T, Eistetter H, Katz M, Schmidt W, Kemler R. The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J Embryol Exp Morphol* 1985; 87:27-45.